Vitamin D Receptor Activation Improves Allergen-Triggered Eczema in Mice

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Atopic dermatitis (AD) is a common chronic inflammatory skin disease that has increased in prevalence over the last several decades in industrialized countries. AD is a multifactorial, heterogenous disease with a variety of defects in the immune system, in antimicrobial defense mechanisms and epidermal barrier integrity, which collectively contribute to the risk and severity of AD development. Vitamin D receptor (VDR) signaling has been shown to be important not only in the immune system but also in the skin and in particular keratinocytes to regulate skin homeostasis and epidermal barrier function. However, this work aimed to analyze the role and clinical efficiency of VDR activation by a VDR agonist without calcium-mobilizing activity in a mouse model of allergen-triggered eczema. We show that the systemic administration of the low-calcemic VDR agonist significantly improved the allergen-triggered eczema. Thereby, *forkhead box P3* (*Foxp3*)-expressing regulatory T cells, revealed to have a role in AD, were selectively increased in the skin of VDR agonist-treated mice. Moreover, our results demonstrate a marked induction of skin barrier gene and antimicrobial peptide gene expression in skin lesions of VDR agonist-treated mice. Thus, our study provides evidence that systemic VDR agonist treatment may improve allergen-triggered eczema *in vivo*.

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INTRODUCTION

1α,25-Dihydroxyvitamin D_3 (calcitriol), the biologically active metabolite of vitamin D_3, has been identified over the past decades to have strong immunomodulatory effects by functioning on cells of the innate and adaptive immune system (Adams and Hewison, 2008; Mora *et al.*, 2008). Calcitriol functions primarily by binding to the vitamin D receptor (VDR), which functions as a ligand-dependent transcription factor (Bouillon *et al.*, 2008). Liganded VDR recruits its preferred dimerization partner, the retinoid X receptor, to form a heterodimer that subsequently binds to vitamin D response elements in the promoter regions of target genes. Similarly, calcitriol and VDR effectively regulate epidermal differentiation and permeability barrier homeostasis (Bikle, 2010).

The prevalence of atopic dermatitis (AD) has increased over the last decades in industrialized countries, now affecting around 15–30% of children and 2–10% of adults (Bieber, 2010). AD is a chronic and relapsing eczematous skin inflammation associated with epidermal barrier dysfunction, intense pruritus, and cutaneous hyperreactivity to environmental triggers (Leung *et al.*, 2004; Bieber, 2008). By histopathology, AD skin lesions display prominent infiltrates of mononuclear cells in the dermis combined with intercellular edema in the epidermis (spongiosis; Bieber, 2008). The exact cause of AD is not well understood, but the disease is regarded as multifactorial, heterogenous, and associated with defects of the immune system, in antimicrobial defense mechanisms and epidermal barrier integrity (De Benedetto *et al.*, 2009; Bieber, 2010).

The permeability barrier function is not regulated by the corneocyte, but rather by the lipid-enriched, extracellular matrix of the stratum corneum, which is mainly composed of the structural proteins, loricrin, involucrin, filaggrin (FLG), and small proline-rich proteins (Scharschmidt *et al.*, 2009). By cross-linking these and other proteins, transglutaminases are similarly involved in the formation of the epidermal barrier (Candi *et al.*, 2005). Recently, loss-of-function genetic variants of the filaggrin gene (*FLG*) encoding the intracellular protein, FLG, have been reported to be strong predisposing factors for AD (Palmer *et al.*, 2006). FLG deficiency alone provokes a permeability barrier abnormality

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Abbreviations: AD, atopic dermatitis; AU, arbitrary unit; CYP27B1 (1αOHase), 25-hydroxyvitamin D_3-1α-hydroxylase; FLG, filaggrin; Foxp3, forkhead box P3; OVA, ovalbumin; T_{eff}, T effector cell; T_H, T helper cell; T_{reg}, regulatory T cell; VDR, vitamin D receptor

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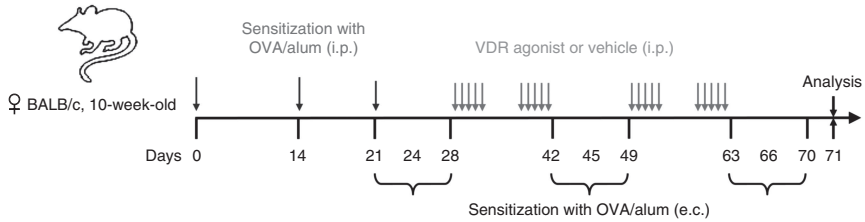

Figure 1. Experimental setup for ovalbumin (OVA) sensitization and vitamin D receptor (VDR) agonist treatment. Mice were sensitized intraperitoneally (i.p.) and epicutaneously (e.c.) as described in Materials and Methods. Treatment with VDR agonist or control started upon removal of the first patch (light gray arrows). On day 71, mice were killed and skin samples collected.

(Scharschmidt *et al.*, 2009). Other reports showed reduced expression of antimicrobial peptides, involucrin, and loricrin in AD skin (De Benedetto *et al.*, 2009).

The importance of VDR signaling for epidermal differentiation and permeability barrier homeostasis has been shown by *in vitro* and *in vivo* studies. For instance, calcitriol has been reported to increase involucrin and transglutaminase expression in keratinocytes (Su *et al.*, 1994; Bikle *et al.*, 2001, 2002). Silencing of the VDR and two VDR coactivators blocked keratinocyte differentiation and decreased the expression of FLG (Oda *et al.*, 2007). VDR-null mice exhibit a defect in epidermal differentiation as shown by reduced levels of involucrin, proFLG, and loricrin, as well as loss of keratohyalin granules (Xie *et al.*, 2002). In addition, CYP27B1 (1αOHase) (25-hydroxyvitamin D_3-1α-hydroxylase)-null animals, which are unable to produce the biologically active calcitriol from its precursor 25-hydroxyvitamin D_3, show a reduction in the epidermal differentiation markers involucrin, FLG, and loricrin and display a markedly delayed recovery of normal barrier function following disruption of the barrier (Bikle *et al.*, 2004).

On the basis of these observations, we hypothesized that specific VDR targeting may represent a therapeutic option for the treatment of AD. Owing to its major function in calcium and phosphorus homeostasis, calcitriol itself, however, has serious side effects (hypercalcaemia, hypercalciuria, and bone resorption), which hamper its clinical use (van Etten and Mathieu, 2005). To circumvent this limitation, synthetic so-called dissociated VDR agonists have been developed. They maintain or even augment the beneficial immunomodulatory effects but display weak calcium-mobilizing activity. Here, we wanted to delineate whether VDR activation might be of therapeutic benefit in the treatment of AD. Therefore, we used a recently described low-calcemic VDR agonist that shows profound immunomodulatory activities (Zugel *et al.*, 2009), in particular with regard to the B-cell-dependent allergic immune response (Hartmann *et al.*, 2011). We show that the VDR agonist induces the expression of genes associated with barrier function and, more importantly, inhibits the development of allergen-triggered eczema in a mouse patch test model.

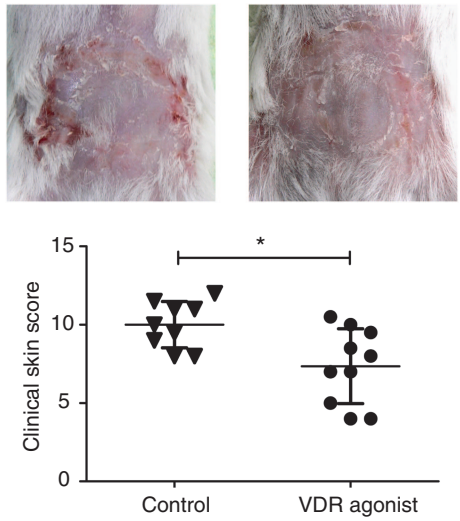

Figure 2. The vitamin D receptor (VDR) agonist inhibits allergen-triggered skin eczema. Mice were sensitized and treated with the VDR agonist or control as described in Figure 1 and Materials and Methods. On day 71, mice were killed and the severity of the eczema was evaluated as described in Materials and Methods. The index of severity (clinical skin score) is shown as mean ± SD for each group ($n \geqslant 9$; $^*P < 0.05$). One representative picture of each group is shown.

RESULTS

The VDR agonist ameliorates allergen-triggered eczema

By using a VDR agonist that does not induce hypercalcemia but nevertheless possesses strong immunomodulatory effects (Zugel *et al.*, 2009), we set out to investigate the influence of VDR signaling in a defined model of allergen-triggered eczema (Figure 1). Therefore, mice were sensitized three times with ovalbumin (OVA) intraperitoneally, followed by the epicutaneous challenge with OVA. Subsequently, mice were treated with either the VDR agonist or PBS control, starting after removal of the first patch to have a therapeutic setting regarding the epicutaneous sensitization. VDR activation by the agonist led to a clear improvement in all clinical signs of the OVA-triggered eczematous response, namely erythema, edema, excoriation, dryness, and extension. Thus, VDR agonist treatment reduced the clinical skin score from 10.0 ± 1.5 to 7.4 ± 2.4 (Figure 2). Taken together, treatment with the low-calcemic VDR agonist leads to a significant improvement of the allergen-triggered eczematous response.

VDR activation does not significantly alter local T-cell infiltration

Immunohistochemistry was performed to analyze the relative distribution of T-cell subsets in lesional skin. As shown in

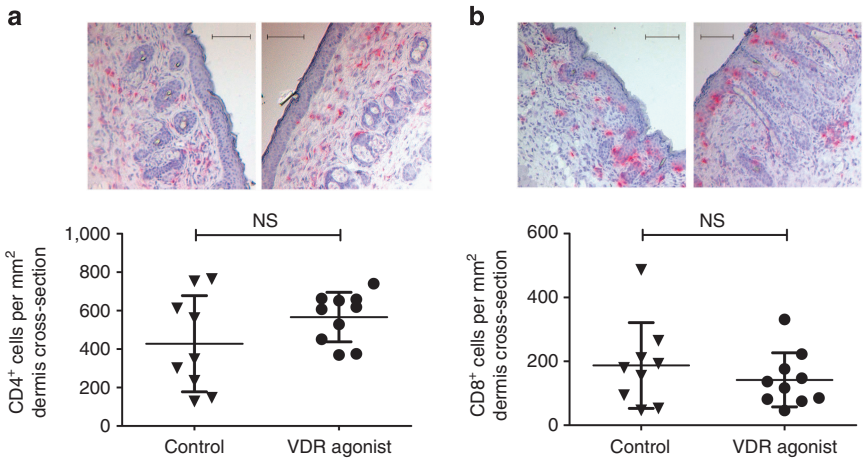

Figure 3. CD4$^+$ and CD8$^+$ cells in skin lesions of ovalbumin (OVA)-sensitized and vitamin D receptor (VDR) ligand-treated mice. CD4$^+$ (**a**) and CD8$^+$ (**b**) T cells were stained and quantified in the dermis of lesional skin of VDR agonist-treated and control mice. Numbers of cells per mm^2 are shown as mean ± SD for each group ($n \geqslant 9$). One representative photograph of each group is shown; NS, not significant. Bar = 100 µm.

Figure 3a, there was no significant alteration between VDR agonist-treated and control mice in terms of total CD4$^+$ T cells. Nevertheless, a tendency of higher CD4$^+$ T-cell numbers was observed in the VDR ligand-treated group (VDR agonist: 566.5 ± 128.9 cells per mm^2; control mice: 427.9 ± 249.7 cells per mm^2; $P = 0.1628$; corresponding to a 1.3-fold increase). Conversely, there was a slight tendency of CD8$^+$ T cells to decrease in the skin of VDR agonist-treated mice, but this did not reach statistical significance (Figure 3b). Taken together, overall T-cell skin infiltrates remain stable upon VDR activation despite the clinical improvement in the signs of eczematous response.

The VDR agonist specifically increases Foxp3$^+$ cells in skin lesions

The above data implied that total CD4$^+$ T-cell numbers in skin lesions were not decreased upon VDR activation, but rather showed the opposite tendency, and that this occurred simultaneously with an improvement of clinical signs. The CD4$^+$ cell compartment comprises a heterogenous group of T cells, including the T helper (T_H), T effector (T_{eff}), and the regulatory T-cell subset, the latter being forkhead box P3 (Foxp3) positive (Zhu and Paul, 2010). We therefore analyzed the density of Foxp3$^+$ cells in lesional skin. It was found that there was indeed a clear-cut increase in the number of Foxp3$^+$ cells by 33.9% upon treatment with the VDR agonist (VDR agonist: 193.7 ± 38.6 cells per mm^2; control: 144.7 ± 43.2 cells per mm^2; $P = 0.026$; Figure 4a), which might explain the increase in infiltrating CD4$^+$ T cells (Figure 3a). The increase in Foxp3$^+$ cells was confirmed by a 2.0-fold increased expression of *Foxp3* mRNA in the skin of VDR agonist-treated mice (VDR agonist: 2.59 ± 1.01 arbitrary unit (AU); control: 1.28 ± 0.91 AU; $P = 0.0148$; Figure 4b). As Foxp3$^+$ regulatory cells function by dampening responses of T_{eff}, and both T_H1 and T_H2 subsets, we also analyzed the skin sections for its T_H1 and T_H2 signature cytokine expression. The VDR agonist reduced *Il4* expression in lesional skin by 32.2% (VDR agonist: 0.63 ± 0.26 AU; control: 0.93 ± 0.30 AU; $P = 0.0435$; Figure 4c). In addition, *Ifng* expression was reduced by 41.8%, but this did not reach statistical significance (VDR agonist: 0.12 ± 0.07 AU; control: 0.20 ± 0.13 AU; $P = 0.0749$; Figure 4d). Taken together, these data show that Foxp3$^+$ cells are selectively increased in the skin after VDR activation.

VDR activation induces barrier and antimicrobial peptide gene expression

VDR ligands are known to induce genes important for skin barrier function (Bikle, 2010), the impairment of which is a hallmark of AD pathogenesis (Cork *et al.*, 2009). We therefore determined whether the VDR agonist may induce barrier and antimicrobial gene expression in lesional skin. The treatment with the VDR agonist indeed resulted in a robust induction of several skin barrier genes, including loricrin (*Lor*), involucrin (*Ivl*), transglutaminase 1 (*Tgm1*), and filaggrin (*Flg*), and, moreover, the antimicrobial peptide β-defensin 2 (*Defb2*) and, to a lesser degree, β-defensin 3 (*Defb3*; Figure 5). The highest induction of gene expression with about 6.1-fold was found for loricrin ($P = 0.0003$) and transglutaminase 1 (4.6-fold, $P < 0.0001$; Figure 5a and c). The induction of FLG (Figure 5d) and involucrin (Figure 5b) by VDR treatment was 3.3-fold ($P < 0.0001$) and 4.4-fold ($P < 0.0001$), respectively. The expression of β-defensin 2 was 4.1-fold ($P = 0.0006$, Figure 5e) and β-defensin 3 was 0.2-fold ($P = 0.0464$, Figure 5f) increased by the VDR agonist compared with the control. Taken together, these data show that VDR ligation induces the expression of skin barrier and antimicrobial peptide genes in eczematous skin lesions.

DISCUSSION

We report here that systemic low-calcemic VDR agonist treatment ameliorates allergen-triggered eczema in mice, a model closely reflecting AD in humans. This study was prompted by our recent observation that the low-calcemic VDR agonist ZK203278 dampens B-cell-dependent allergic

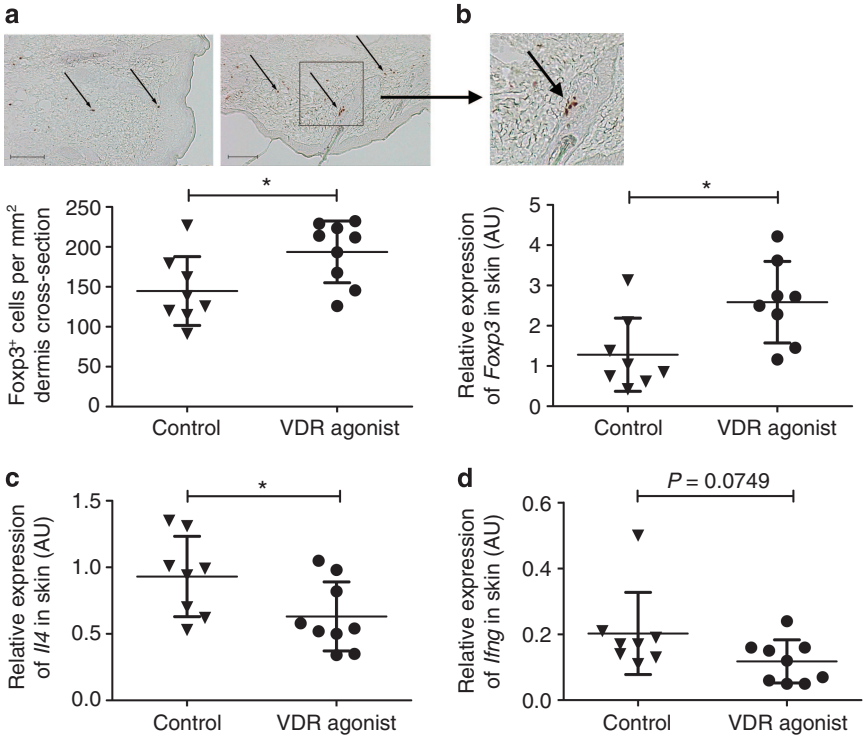

Figure 4. The vitamin D receptor (VDR) agonist increases the number of Foxp3$^+$ cells in lesional skin. (**a**) Foxp3$^+$ cells were stained and quantified in the dermis of lesional skin of VDR agonist-treated and control mice. Numbers of cells per mm^2 are shown as mean ± SD for each group ($n \geq 8$; *$P < 0.05$). One representative photograph of each group is shown; bar = 100 μm. Expression levels of *Foxp3* (**b**), *Il4* (**c**), and *Ifng* (**d**) in lesional skin of each group were assessed by quantitative PCR. Shown is the relative expression of the target gene compared with the housekeeping gene *Hprt* in arbitrary units (AU; $n \geq 8$; *$P < 0.05$). The arrows in panels **a** and **b** denote the Foxp3$^+$ cells.

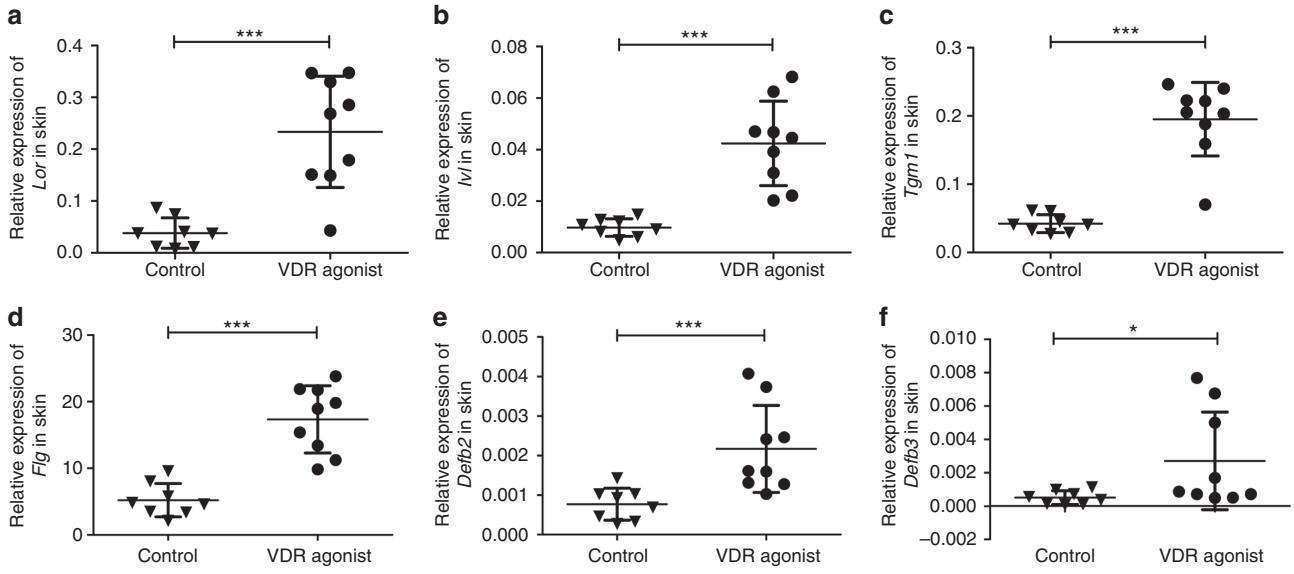

Figure 5. Systemically applied vitamin D receptor (VDR) agonist induces barrier and antimicrobial genes in lesional skin. Expression levels of skin barrier genes and antimicrobial peptides in lesional skin were analyzed by quantitative PCR. The relative expression of loricrin (*Lor*, **a**), involucrin (*Ivl*, **b**), transglutaminase 1 (*Tgm1*, **c**), filaggrin (*Flg*, **d**), β-defensin 2 (*Defb2*, **e**), and β-defensin 3 (*Defb3*, **f**) was measured in comparison with the housekeeping gene *Hprt* and is shown as mean ± SD for each group ($n \geq 8$; *$P < 0.05$, ***$P < 0.001$).

immune responses *in vitro* and *in vivo* (Hartmann *et al.*, 2011), as well as by reports about its role in keratinocyte differentiation (Bikle, 2004, 2010). In fact, a clear-cut improvement in the signs of the eczematous response, as summarized in the clinical skin score, was detected upon treatment with the VDR agonist.

Dermal CD4$^+$ T-cell infiltration is critical for the development of AD skin lesions (Oyoshi *et al.*, 2009).

By analyzing VDR-mediated cellular changes, we were able to observe a minor increase in the number of infiltrating $CD4^+$ T cells upon VDR activation, simultaneously with an improvement of the skin. We therefore reasoned that the VDR agonist might have altered the composition of $CD4^+$ T-cell subsets in the skin. T_H cells consist of several subsets, each of which impact immune responses in a unique manner (Zhu and Paul, 2010). Natural $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (T_{regs}), which comprise 5–10% and 1–2% of the $CD4^+$ T-cell pool in mice and humans, respectively (Ghoreishi *et al.*, 2009), and other subtypes of induced T_{reg} cells inhibit both T_H1- and T_H2-cell function by reducing their cytokine production and by direct interaction with effector T cells (Beissert *et al.*, 2006). T_{reg} cells are crucial for the maintenance of skin immune homeostasis (Dudda *et al.*, 2008), and their number is strongly decreased in inflammatory skin diseases, including AD and psoriasis (Verhagen *et al.*, 2006). In the present study, we found increased numbers of $Foxp3^+$ cells in VDR agonist-treated mice and this was associated with reduced expression of *Il4* and *Ifng* in the skin. The importance of VDR signaling for proper T_{reg} function in the skin is underlined by the finding that VDR-knockout mice fail to increase $Foxp3^+$ T_{regs} in skin-draining lymph nodes following UV irradiation (Ghoreishi *et al.*, 2009). There is other robust evidence for a crucial role of the VDR for T_{reg} generation, maintenance, and function. For example, a combination of calcitriol and dexamethasone or calcitriol alone has been shown to induce $CD4^+$ T cells to differentiate *in vitro* into T_{reg} cells, even in the absence of antigen-presenting cells (Barrat *et al.*, 2002; O'Garra and Barrat, 2003; Jeffery *et al.*, 2009). Moreover, topical calcitriol or calcipotriol treatment was shown to enhance the suppressive activity of T_{regs} in skin-draining lymph nodes, whereas calcipotriol caused the expansion of antigen-specific T_{reg} cells (Gorman *et al.*, 2007; Ghoreishi *et al.*, 2009). Taken together, the enhanced suppressive T_{reg} function likely contributes to the VDR-mediated improvement of allergen-triggered eczema.

Li *et al.* (2006) reported that topical treatment with calcitriol and MC903 (a VDR agonist) induces thymic stromal lymphopoietin and triggers an AD-like phenotype. In their model, topical application of 0.1–0.17 μg calcitriol was applied per ear. In the current study, only 0.15 μg of the VDR agonist was applied systemically via intraperitoneal injections. Thus, it cannot be assumed that high local concentration as in the study by Li *et al.* was achieved in our setting. However, we observed an increase in *Tslp* expression in skin samples from mice that were systemically treated with the VDR agonist (Supplementary Figure S1 online). In contrast, we did not observe an aggravation of skin eczema in these mice, which may result from counter-regulating mechanisms, in particular by similar effects on skin barrier gene expression and regulatory T cells. In addition, topically applied calcitriol and analogs represent successful therapeutic approaches in psoriasis (Sigmon *et al.*, 2009). In fact, calcitriol at 3 $\mu g\,g^{-1}$ ointment was found to be a safe and effective treatment for mild-to-moderate psoriasis with only mild local adverse effects in a small number of patients (Sigmon *et al.*, 2009). Clearly, psoriasis patients treated with VDR agonists do not normally develop AD or AD-like lesions. Conversely, it is now well recognized that psoriasis and AD share many features, despite evident differences, and are more strongly related than previously suspected. For example, both display mutations in partially common genes and are characterized by dysregulated T-cell responses and profound barrier defects (Bieber, 2008, 2010; De Benedetto *et al.*, 2009; Nestle *et al.*, 2009; Roberson and Bowcock, 2010). The correct formation and function of the epidermal barrier are ensured by the expression of proteins such as FLG, involucrin, loricrin, and transglutaminases, and a complex series of lipids (Candi *et al.*, 2005). Perturbed barrier function in AD has been reported in mouse and man. For example, NC/Nga mice, a spontaneous mouse model of AD, show skin barrier abnormalities under conventional conditions (Aioi *et al.*, 2001). Flaky tail mice (*ft/ft*) are characterized by dry, flaky skin associated with increased transepidermal water loss, as observed in patients with AD and FLG mutations (Presland *et al.*, 2000; Kezic *et al.*, 2008). In addition, loss-of-function genetic variants of the *FLG* gene have been reported to be strong predisposing factors for AD (Palmer *et al.*, 2006; Weidinger *et al.*, 2006; O'Regan and Irvine, 2008), whereas others showed a reduced expression of antimicrobial peptides, involucrin, and loricrin in AD skin (Kim *et al.*, 2008; De Benedetto *et al.*, 2009). We have found that VDR activation clearly improved the signs of allergen-triggered eczema, simultaneously with a strong increase in the expression of genes important for the permeability barrier function such as FLG, loricrin, involucrin, and transglutaminase I, but also of the antimicrobial peptides β-defensin 2 and 3. This is in line with previous reports showing a direct increase in involucrin and transglutaminase expression by VDR activation in keratinocytes (Su *et al.*, 1994; Bikle *et al.*, 2001, 2002). Similarly, silencing of the VDR and VDR coactivators led to decreased FLG expression and subsequently decreased keratinocyte differentiation (Oda *et al.*, 2007). Moreover, data from $VDR^{-/-}$ and $CYP27B1^{-/-}$ ($1\alpha OHase^{-/-}$) mice exhibit a defect in epidermal differentiation as shown by reduced levels of involucrin, FLG, and loricrin (Xie *et al.*, 2002; Bikle *et al.*, 2004). In accordance with previous reports showing the crucial role of IL-4 for regulating epidermal homeostasis and innate barrier function (Kim *et al.*, 2008; Sehra *et al.*, 2010), reduced *Il4* expression as observed in VDR agonist-treated mice may have contributed to the upregulation of genes forming the cornified envelope. In addition, Hong *et al.* (2008) have shown increased mouse β-defensin 2 and 3 expression at both the mRNA and protein level after 3 days of UVB exposure in parallel with a beneficial effect on permeability barrier function, which depends, at least in part, on activation of the cutaneous vitamin D system. Importantly, *Cyp24a1* expression, the most sensitive indicator for VDR activation (Schuster, 2011), was shown to be markedly increased in mouse skin between 4 and 6 hours after intraperitoneal administration with a pharmacological dose of calcitriol (Akeno *et al.*, 1997).

Taken together, our study has provided evidence that systemic low-dose treatment with a low-calcemic VDR

agonist may improve allergen-induced eczema *in vivo*, which to our knowledge has not been previously reported. Therefore, targeting the VDR with low-calcemic agonists may develop into a feasible treatment option for AD. Future studies in humans will be required to assess the full potential of VDR-targeted therapies for AD management.

MATERIALS AND METHODS

Mice and treatments

Female, 10-week-old BALB/c mice (Federal Institute of Risk Assessment, Berlin, Germany) were maintained in a pathogen-free environment under laboratory conditions with mouse chow and water available *ad libitum*. All experimental procedures were performed in compliance with protocols approved by the local State Office of Health and Social Affairs. Mice were sensitized intraperitoneally using 10 μg OVA (Sigma-Aldrich, Steinheim, Germany) adsorbed to 1.5 mg aluminum hydroxide and magnesium hydroxide (Imject Alum, Thermo Fisher Scientific, Schwerte, Germany) in a total volume of 100 μl on day 1, 14, and 21 (Figure 1).

For the induction of AD skin lesions, the epicutaneous allergen application patch test was used (Dahten *et al.*, 2008); the scheme is depicted in Figure 1. Mice were shaved on the ventral side with a wet razor. A measure of 100 μg OVA (Sigma-Aldrich) adsorbed to 1.5 mg aluminum hydroxide and magnesium hydroxide (Imject Alum, Thermo Fisher Scientific) in a total volume of 20 μl was applied to a sterile patch and secured to the skin with an elastic cohesive bandage. The patch was placed for three 1-week periods (with patch renewal after 3 days) separated by a 2-week interval between each application. ZK203278 (hereinafter referred to as VDR agonist; 6 $\mu g\,kg^{-1}$ per day in PBS/0.03% ethanol) or an equal volume of PBS/0.03% ethanol as control was injected intraperitoneally for two consecutive 5-day periods separated by a 2-day-break after the first and second patch (Figure 1).

Assessment of allergen-triggered eczema

Erythema, edema, excoriation, dryness, and extension were used as typical hallmarks of human AD to evaluate clinical severity as described previously (Dahten *et al.*, 2008; Babina *et al.*, 2010). Each parameter was evaluated independently and blindly by six investigators. Severity was rated according to scores as follows: 0, no reaction observed; 1, mild signs; 2, intermediate signs; 3, severe signs. The total score was taken as the index of severity (clinical skin score).

Immunohistochemistry

Sections of 5 μm were prepared and used for staining by the streptavidin–biotin complex system as described previously (Babina *et al.*, 2010). Briefly, sections were blocked with 10% normal goat serum (Dako, Hamburg, Germany) and the avidin/biotin blocking kit (Vector Peterborough, Cambridgeshire, UK), and then incubated with rat anti-mouse CD4 (RM4–5) or CD8 (53-6.7) followed by incubation with biotinylated goat anti-rat IgG (all from BD Pharmingen, Heidelberg, Germany). Negative controls were run in parallel omitting either the primary or the secondary antibody. Signals were detected by the alkaline phosphatase/red detection kit and by hematoxylin counter staining. $Foxp3^+$ cells were stained as described (Heimesaat *et al.*, 2007). In all cases, positively stained cells were counted at a ×100 magnification in 3–6 fields of $200 \times 200\,\mu m^2$, and are expressed as mean cell number per mouse normalized to $1\,mm^2$.

RNA isolation from skin samples

Samples from lesional skin (5 μm) were collected with a punch biopsy and frozen instantly in liquid nitrogen, and stored at −80 °C. Samples were homogenized by pounding in a mortar in liquid nitrogen. Homogenized samples were subjected to proteinase K (Macherey-Nagel, Düren, Germany) digestion for 10 minutes at 55 °C. For RNA isolation, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol with an additional step of DNase (Macherey-Nagel) digestion for 15 minutes at room temperature. The quality of the RNA samples was assessed by agarose gel electrophoresis upon ethidium bromide staining (Sigma-Aldrich, Steinheim, Germany).

Reverse transcription and quantitative PCR

RNA was reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) and quantitative PCR performed with the Fast Start DNA Master SYBR Green I and LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Oligonucleotide primers were synthesized by TIB MOLBIOL (Berlin, Germany) and are listed in Supplementary Table S1 online. The expression levels of target gene mRNA were quantified relative to the expression of the reference gene *Hprt*. Relative gene expression was calculated by the comparative ΔC_T method as described (Schmittgen and Livak, 2008).

Data analysis

Data were analyzed using the statistical program GraphPad Prism (GraphPad Software) by applying an appropriate test (unpaired *t*-test or non-parametric Mann–Whitney *U*-test when data were not normally distributed). Data are shown as mean ± SD. Significance between experimental and control mice was assumed at $P<0.05$ for all tests.

CONFLICT OF INTEREST

The authors UZ and AS are employees and shareholders of Bayer. The patent rights for ZK203278 are held by Bayer.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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